INCORPORATION OF [1-^{14}C]ACETATE INTO THE ALIPHATIC δ-LACTONES OF RUMINANT MILK FAT*

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SUMMARY

1. Gas–liquid radiochromatography was employed to demonstrate that ^{14}C from [1-^{14}C]acetate was incorporated *in vivo* into the aliphatic δ-lactones (δ-hydroxy fatty acids) of goat milk fat.

2. The δ-C_{10}, δ-C_{12}, δ-C_{14} and δ-C_{16} lactones were isolated from milk fat samples collected at 5, 10, 15, 20 and 25 h following intravenous and intramammary administration of [1-^{14}C]acetate to two goats and the specific activities of the δ-lactones were determined. These values, in general, were of comparable magnitude to the specific activities of the saturated milk fatty acids of corresponding carbon chain lengths.

3. Following intravenous administration of [1-^{14}C]acetate both the δ-lactones and the saturated fatty acids exhibited maximum specific activities at 5 h whereas following intramammary administration the δ-lactones and fatty acids exhibited maximum specific activities at 10 h.

4. The results are consistent with the view that acetate is a common precursor of both the δ-lactones (δ-hydroxy fatty acids) and the saturated fatty acids and imply that the δ-hydroxy fatty acids are produced from acetate in a manner similar to biosynthesis of the corresponding fatty acids rather than as products of long chain fatty acid oxidation.

INTRODUCTION

When freshly secreted milk fat is subjected to heat treatment or storage, an homologous series (C_6–C_{18}) of saturated aliphatic δ-lactones is produced[1–4]. The total quantity produced in ruminant milk fat is variable but in the range 50–150 μg/g fat. The δ-C_{10}, δ-C_{12}, δ-C_{14} and δ-C_{16} lactones together constitute about 90% of this total[5,6].

Several studies have demonstrated that the precursors of the δ-lactones in fresh milk fat are the corresponding δ-hydroxy fatty acids esterified to glycerol[7–9]. Lactones

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are formed from these precursors by hydrolysis of the unstable hydroxy acid ester bond and subsequent loss of water from the liberated δ-hydroxy fatty acids.

BOLDINGH *et al.*[10] established the biological origin of the δ-hydroxy fatty acids by the discovery that they exist in only one of two possible optically active forms. Furthermore, the lack of evidence to demonstrate the presence of a series of δ-lactones in the non-oxidized oils of plants generally consumed by ruminants suggests that the δ-hydroxy fatty acids are products of endogenous animal lipid metabolism. This suggestion is substantiated by the studies of VIRTANEN[11] who reported that δ-lactones are present in the milk fat of cows fed protein-free diets which contain small amounts of pure vegetable oils to at least the same levels as in the milk fat of control cows fed normal diets.

DIMICK *et al.*[12] have reported that δ-lactones occur in trace quantities in the milk fat of monogastric animals and in the adipose tissue of both ruminants and non-ruminants which tends to preclude the rumen and the mammary gland as exclusive sources of the δ-hydroxy fatty acids. The authors speculated that biosynthesis of these δ-hydroxy acids may occur at all sites of lipogenesis within an animal, but to the greatest extent in tissues where this process is optimum, *e.g.*, in the mammary gland.

In a recent study DIMICK AND HARNER[13] investigated the environmental and physiological factors which influence the δ-lactone potential of bovine milk fat. The results demonstrated a highly significant positive correlation between amounts of δ-lactone and esterified short-chain (C_4–C_{14}) saturated fatty acids in milk fat samples from an individual cow taken weekly during 310 days of lactation. In addition dramatic decreases were observed in both the δ-lactone and short-chain fatty acid content of the milk fat from cows which exhibited clinical symptoms of bovine ketosis. These correlations encouraged speculation concerning a relationship between the biosynthesis of δ-hydroxy fatty acids and the *de novo* biosynthesis of saturated (C_4–C_{16}) fatty acids from acetate.

The present investigation was undertaken to determine whether the ^{14}C from [1-^{14}C]acetate is incorporated *in vivo* into the δ-lactones of goat milk fat and, if so, to measure the specific activities of both the δ-lactones and the saturated fatty acids in milk fat collected following administration of [1-^{14}C]acetate with the expectation that comparison of the labeling patterns may yield some information concerning the pathway of biosynthesis of δ-hydroxy fatty acids in the ruminant.

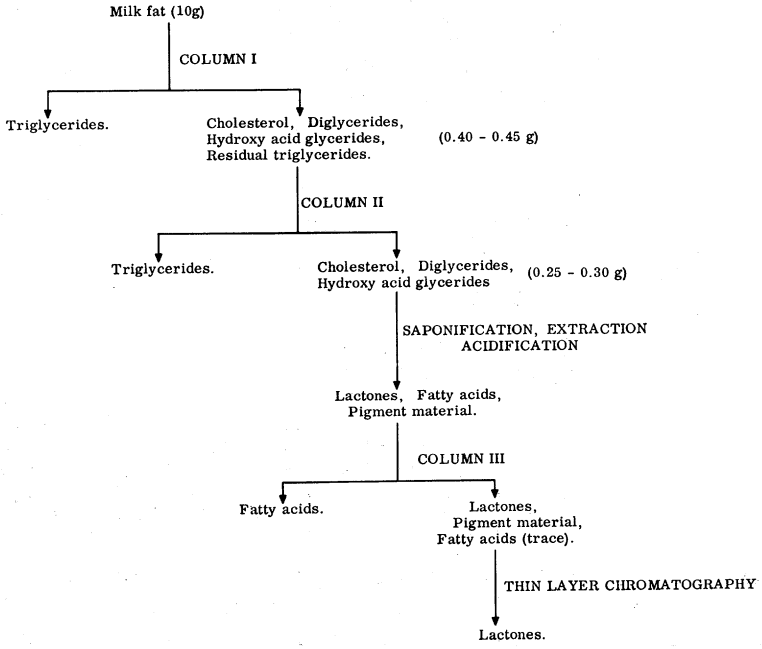
EXPERIMENTAL PROCEDURE

Two healthy lactating goats, each producing approx. 1500 ml of milk per day were used in this study. After complete milking, goat A received 1.0 mC of [1-^{14}C]-acetate *via* injection into the jugular vein, and goat B received 1.0 mC of [1-^{14}C]acetate *via* intramammary infusion into both halves of the udder. To obtain approx. 10 g fat from each milking the goats were milked at 5, 10, 15, 20 and 25 h following administration of the radioactive substrate. The fat was recovered from the milk samples by the Roese–Gottlieb solvent extraction method[14]. The specific activities were determined by assay in a Packard Tri-Carb liquid scintillation spectrometer.

Isolation and quantitative analysis of the δ-lactones

Silicic acid adsorption chromatography was employed to isolate the lactone-rich

fractions from the radioactive milk fat samples. A scheme illustrating the steps employed in the isolation procedure is presented in Scheme I. The procedure has been described by DIMICK *et al.*[12]. Further purification by thin-layer chromatography was achieved by streaking the lactone-rich fraction across the origin of a plate (20 cm × 20 cm) spread with silica gel H to a thickness of 0.4 mm. The plate was developed



Scheme I. Scheme illustrating the procedure employed to isolate a lactone-rich fraction from milk fat.

in a sealed tank with a solvent system consisting of light petroleum (b.p., 30–60°)–diethyl ether–acetic acid (70:30:1, v/v/v). The band containing the δ-lactones was located by spraying a side strip with a 2% (w/v) solution of iodine in methanol. This band was scraped from the plate and eluted with diethyl ether.

A Barber–Colman model 10 gas chromatograph, equipped with an argon ionization detector was employed for quantitative analysis of the individual δ-lactones[15]. Calibration curves relating gas chromatographic peak areas to known concentrations of authentic δ-lactones were prepared for use in the analyses. A typical chromatogram is presented in Fig. 1.

Gas chromatography was also employed to isolate individual δ-lactones from the lactone-rich fractions by trapping from the carrier gas effluent. Trapping was achieved by attaching a 6 inch × 0.25 inch Z-shaped glass tube filled with washed sand, *via* a hypodermic needle to the heated (250°) outlet of the column. The traps were subsequently eluted with diethyl ether from a syringe. Aliquots of the δ-C_{12} and δ-C_{14} lactones were subsequently injected into a Barber-Colman model 5000 gas chromatograph equipped with a model 5190 radioactivity monitoring system for detection of organic ^{14}C as $^{14}CO_2$ (see ref. 16).

Determination of specific activities

For determination of the specific activities of the δ-lactones a known aliquot of the lactone-rich fraction was injected into the gas chromatograph and components in each of the fractions designated in Fig. 1 were successively trapped from the carrier gas effluent by the procedure described previously. In this case the traps were eluted directly into vials with three 5-ml quantities of scintillation liquid for radioactivity assay.

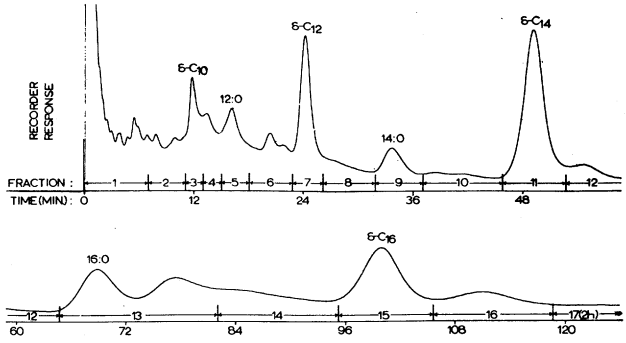


Fig. 1. A typical gas–liquid chromatogram of a lactone-rich fraction isolated from milk fat, showing the fractions which were individually trapped from the carrier gas effluent. Column: 6 ft × 0.25 inch, U-shaped glass packed with 10% (w/w) diethyleneglycol adipate + 2% (w/w) phosphoric acid on 80/100 mesh Gas Chrom A. Operating conditions: column temp., 185°; detector temp., 235°; flash heater, 250°; carrier gas (argon) pressure, 20 lb/inch²/g and detector voltage, 750 V.

Gas–liquid radiochromatography[16] was employed for determination of the specific activities of the fatty acids in each of the radioactive milk fat samples. Methyl esters of the fatty acids were prepared by the procedure of METCALFE, SCHMITZ AND PELKA[17]. A typical gas–liquid radiochromatogram is presented in Fig. 2.

RESULTS AND DISCUSSION

The specific activities of the milk fats collected from the two goats following administration of [1-^{14}C]acetate are listed in Table I. Incorporation of ^{14}C into the total milk fat collected from each goat was 6% following intravenous injection and 16% following intramammary infusion.

TABLE I

SPECIFIC ACTIVITIES OF THE MILK FATS FROM GOATS A AND B

Goat A received 1.0 mC of [1-^{14}C]acetate by intravenous injection and goat B the same by intramammary infusion.

Tima after administration (h)	*Specific activity (counts/min per mg)*	
	Goat A	*Goat B*
5	2808	4334
10	2372	6397
15	820	2725
20	337	1281
25	139	643

The average concentrations of the δ-C_{10}, δ-C_{12}, δ-C_{14} and δ-C_{16} lactones recovered from the milk fat of goats A and B are presented in Table II. The trace quantities of δ-C_6 and δ-C_8 lactones occurring in ruminant milk fat were not recovered from the goat milk fat samples in sufficient amounts to permit an accurate determination of

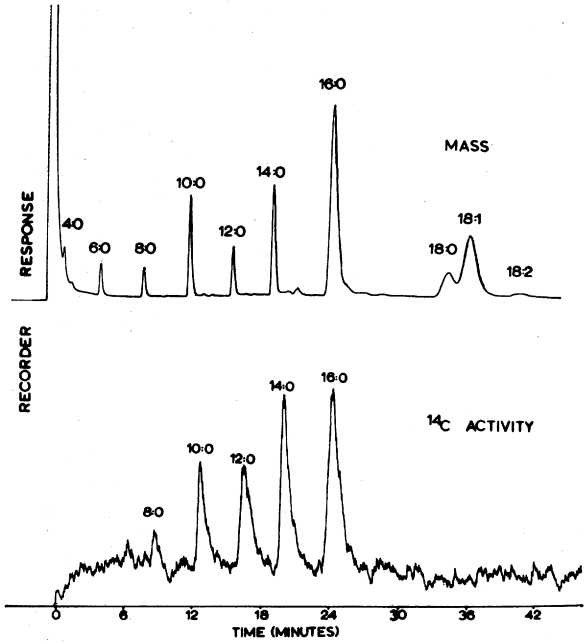


Fig. 2. A typical gas–liquid radiochromatogram showing distribution of ^{14}C in the fatty acids of milk fat following administration of [1-^{14}C]acetate to a goat. Column: 6 ft × 0.25 inch, U-shaped glass packed with 10% (w/w) diethyleneglycol adipate + 2% (w/w) phosphoric acid on 60/80 mesh Gas Chrom P. Operating conditions: column temp., programmed at 6°/min from 75 to 185°; detector temp., 240°; flash heater, 255°; carrier gas (helium) flow rate, 120 ml/min; operating voltage for proportional counter, 1800 V; quench gas (propane) flow rate, 14 ml/min; and combustion tube (CuO) temp., 650°.

concentration. In addition, despite the report by WYATT, PEREIRA AND DAY[8] of the presence of δ-C_{18} lactone in milk fat (approx. 18 mole % of the total δ-lactones) this compound was not detected at any significant levels in the goat milk fat samples.

TABLE II

AVERAGE CONCENTRATIONS OF THE δ-LACTONES RECOVERED FROM THE MILK FAT OF GOATS A AND B

The δ-lactones were isolated from the milk fat by silicic acid adsorption chromatography and thin-layer chromatography. Quantitative analysis of the individual δ-lactones was achieved by gas–liquid chromatography using standard calibration curves. Operating conditions and column packaging for the Barber–Colman model 10 gas chromatograph are those described in the legend for Fig. 1.

Lactone	*Concn. (μg/g)*	
	Goat A	*Goat B*
δ-C_{10}	4.0	2.5
δ-C_{12}	13.0	10.5
δ-C_{14}	26.5	24.5
δ-C_{16}	29.5	26.0

Fig. 3 illustrates the gas–liquid radiochromatograms of the δ-C_{12} and δ-C_{14} lactones isolated from the milk fat of goat B following intramammary infusion of [1-^{14}C]acetate. The results positively establish that the δ-lactones (hence the δ-hydroxy fatty acids) in milk fat do become isotopically labeled following administration of [1-^{14}C]acetate to a goat.

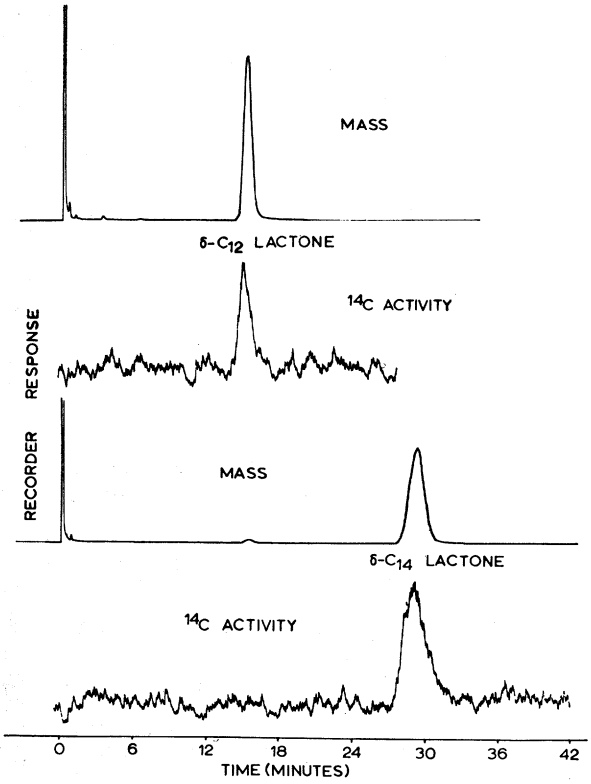


Fig. 3. Gas–liquid radiochromatograms illustrating the presence of ^{14}C in the δ-C_{12} and δ-C_{14} lactones of goat milk fat following intramammary infusion of [1-^{14}C]acetate. The column and operating conditions are the same as those described in the legend for Fig. 3 except that the column temp. was isothermal at 195°.

Specific activities of the δ-lactones and fatty acids

The trapping procedure employed to isolate known quantities of δ-lactone for radioactivity assay proved satisfactory for measurement of specific activities. In all cases recovery of injected ^{14}C in the combined trapped fractions was greater than 85%. No constant bleed of activity was observed and fractions trapped between fatty acid and δ-lactone peaks (Fractions 2, 4, 6, 8, 10, 12, 14 and 16 as designated in Fig. 1) contained less than 10% of the ^{14}C activity in fractions which contained δ-lactones and fatty acids.

Figs. 4 and 5 illustrate the trends in the specific activities of individual δ-lactones and the corresponding fatty acids as a function of time following intravenous injection and intramammary infusion of [1-^{14}C]acetate to goats A and B. Maximum specific activities are observed in individual δ-lactones and corresponding fatty acids

at 5 h following intravenous injection and 10 h following intramammary infusion of [1-^{14}C]acetate. These trends are also observed in the activities of the total milk fats (Table I).

The specific activities of the fatty acids were calculated from gas–liquid radiochromatograms as exemplified in Fig. 2. In many respects the results are in accord

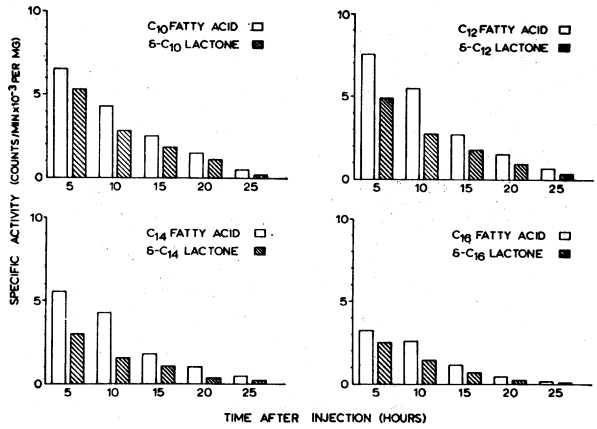


Fig. 4. Relationship between the specific activities of the δ-lactones and the corresponding fatty acids in goat milk fat at 5, 10, 15, 20 and 25 h following intravenous injection of [1-^{14}C]acetate.

with those of other workers who have studied, *in vivo*, the biosynthesis of fatty acids from acetate in the mammary gland of the ruminant[18,19]. An evaluation of the gas–liquid radiochromatograms indicated that the specific activities of the short-chain fatty acids increased as the chain length increased up to C_{10}. However, the specific activities of the C_{10}, C_{12} and C_{14} saturated fatty acids were of similar magnitude at any specific time following administration of [1-^{14}C]acetate and were of greater magnitude than the specific activities of the other fatty acids. This observation suggests that

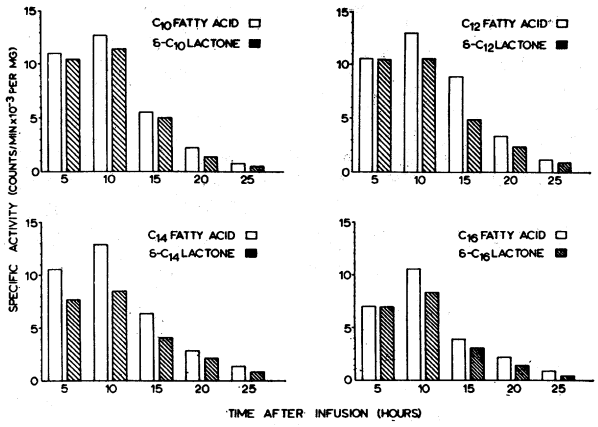


Fig. 5. Relationship between the specific activities of the δ-lactones and the corresponding fatty acids in goat milk fat at 5, 10, 15, 20 and 25 h following intramammary infusion of [1-^{14}C]acetate.

the C_{10}, C_{12} and C_{14} fatty acids in milk fat are derived primarily from acetate in the mammary gland with minimum dilution from other sources.

The specific activity of palmitic acid was lower than those of the C_{10}, C_{12} and C_{14} acids. Unlabeled palmitate taken up from the blood would account for the observed dilution of activity in palmitate synthesized from [1-^{14}C]acetate in the mammary gland. In this respect DIMICK, MCCARTHY AND PATTON[20] and ANNISON *et al.*[21] have reported on the significant contribution of blood plasma palmitate to milk fatty acids in the ruminant.

Further evaluation of the gas–liquid radiochromatograms revealed that the C_{18} acids, even though they constituted 35–45% by weight of the total milk fatty acids, were not isotopically labeled to any significant extent up to 25 h following administration of [1-^{14}C]acetate. This observation is consistent with the view that the major part of the C_{18} milk fatty acids are derived from the blood plasma lipids and that chain elongation of C_{16} acids in the mammary gland constitutes only a minor source of the C_{18} fatty acids[22].

The observed lack of labeling in the C_{18} fatty acids when considered together with relatively comparable specific activities between the δ-lactones and the corresponding fatty acids precludes the possibility the δ-hydroxy fatty acids are formed to any significant extent by oxidative degradation of the C_{18} saturated or unsaturated fatty acids. Furthermore, although some differences are observed in the specific activities and labeling patterns of the δ-lactones and the corresponding fatty acids, the results in general are consistent with the view that acetate is a common precursor of both classes of compounds and that biosynthesis of the δ-hydroxy acids is closely related to the biosynthesis of the corresponding saturated fatty acids.

Complete elucidation of the actual pathway of δ-hydroxy fatty acid biosynthesis cannot be realized from the results of this study. It would seem feasible that in mammary gland tissue, where fatty acid biosynthesis from acetate is intense, small amounts of δ-hydroxy fatty acids may be produced as side-products during the normal biosynthetic process. Alternatively the δ-hydroxy acids may be products of a slightly different biosynthetic pathway involving, perhaps, the incorporation of a C_4 unit into the δ-hydroxy acid chain. To resolve such questions, more detailed study of the labeled δ-lactones is necessary, involving experimental procedures made complex by virtue of the very small quantities (50–150 μg/g) of these compounds potentially present in milk fat.

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